Enzymatic replacement *in vitro* of the first anticodon base of yeast tRNA\(^{Asp}\): application to the study of tRNA maturation *in vivo*, after microinjection into frog oocytes

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**ABSTRACT.**

A combination of several enzymes, RNase-T\(_1\), nuclease S\(_1\), T\(_4\)-polynucleotide kinase and T\(_4\)-RNA ligase were used to prepare and modify different fragments of yeast tRNA\(^{Asp}\) (normal anticodon G U C). This allowed us to reconstitute, *in vitro*, a chimeric tRNA that has any of the four bases G, A, U or C**, as the first anticodon nucleotide, labelled with \((32p)\) in its 3' position.

Such reconstituted \((32p)\) labelled yeast tRNA\(^{Asp}\) were microinjected into the cytoplasm or the nucleus of the frog oocyte and checked for their stability as well as for their potential to work as a substrate for the maturation (modifying) enzymes under *in vivo* conditions.

Our results indicate that the chimeric yeast tRNA\(^{Asp}\) were quite stable inside the frog oocyte. Also, the G\(_{34}\)** was effectively transformed inside the cytoplasm of frog oocyte into Q\(_{34}\) and mannosyl-Q\(_{34}\); U\(_{34}\) into mcm5\(_2\)U and mcm5\(_5\)U. In contrast, C\(_{34}\) and A\(_{34}\) were not transformed at all neither in the cytoplasm nor in the nucleus of the frog oocyte.

The above procedure constitutes a new approach in order to detect the presence of a given modifying enzyme inside the frog oocyte; also it provides informations about its cellular location and possibility about its specificity of interaction with foreign tRNA.

**INTRODUCTION.**

A classical approach in structure-function studies of a tRNA molecule is the isolation and characterization of mutants affected in the corresponding tRNA genes. Identification of the mutants however requires selection of a phenotype *in vivo* and/or availability of a functional test *in vitro*, thus limiting the selection to only those mutants that are not lethal or even that have a definite selective advantage.

This is, for example, the case of nonsense suppressor mutants that can specifically abrogate the effects of nonsense mutations in a cell. (For a review see (1, 2) and references therein).

The recent development of recombinant DNA technology and of rapid DNA sequence analysis has considerably increased the potentiality of a
more direct approach: it is now possible to isolate almost any gene of interest, to modify it by restructuration or by site directed mutagenesis in vitro; and to reinsert it into cells where its functional potential can be measured. A very elegant work of this sort is the construction of a composite tRNA gene by anticodon loop transplant, thus generating new tRNA species that do not occur naturally (3).

Again, while theoretically any chimeric tRNA gene can be prepared in vitro, the cloning step will still be possible only provided that the new tRNA species is not deleterious to the cell. Moreover, it has now become clear that many base or sequence alterations within the coding regions of a tRNA gene, especially from eukaryotic origin, prevent maturation of tRNA, because of impaired initiation of transcription or early processing steps (4-11, the list of references is not exhaustive).

In order to produce certain chimeric tRNAs for testing specific functions other than transcription or processing, it is therefore necessary to develop another methodology that allows to work directly on the mature tRNA transcription product. By using a combination of chemical synthesis and of T₄-RNA ligase and T₄-polynucleotide kinase treatments, it has been possible to reconstitute chimeric or authentic tRNA molecules (12-16).

In the present work we demonstrate the feasibility of performing site specific alterations at the first position of the anticodon in yeast tRNA^Asp. The chimeric tRNA was microinjected into frog oocytes and checked for its potentiality to act as substrate for the oocyte modifying enzymes. Such experiments provide informations additional to those derived from microinjection of tRNA genes into the nucleus of the frog oocytes (8,10, 17-19).

EXPERIMENTAL PROCEDURE.

Materials.

tRNA^Asp from brewer's yeast was obtained as described earlier (20); its acceptor activity for aspartic acid was 1200 pmol/A260 unit. Enzymes used in the course of this study were as follows. RNAase-T₁ (EC 3.1.27.3) and nuclease-S₁ (EC 3.1.30.1) from Aspergillus oryzae (Sigma Chemicals, St-Louis, Missouri); RNAase-T₂ (EC 3.1.27.1) from A. oryzae and nuclease-P₁ (EC 3.1.4) from Penicillium citrinum; RNA ligase (EC 6.5.1.3) from T₄-infected E. coli cells (P.L. Biochemicals,
Milwaukee, Wisconsin); polynucleotide kinase (EC 2.7.1.78) from T₄-infected E. coli cells (Boehringer Mannheim); [γ-³²P] ATP (specific activity higher than 3000 Ci/m mole) was purchased from the Radiochemicals Center Amersham-England. The isomers 2', 3' monophosphates of each of the four nucleosides (Np) were from P.L. Biochemicals. The [5'-³²P] pNp were prepared from the Np and [γ-³²P] ATP using the T₄-polynucleotide kinase as described by England et al. (21), except that 150 units of enzyme/ml were used. Adenosine 5'-triphosphate (ATP); Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid); CHES (2-[N-cyclohexylamino] ethane sulfonic acid) and cacodylic acid (dimethyl arsenic acid) were from Sigma Chemicals, St-Louis, Missouri.

Imidazole, acrylamide and bis-acrylamide, recrystallized by precipitation in acetone, were purchased from Fluka (Switzerland). Cellulose thin layer plates Polygram CEL-300 (20x20x0.1) cm were obtained from Macherey-Nagel and Co, West-Germany.

Preparation of the fragments from yeast tRNA⁰sp.

T₁-hydrolysis.

Under mild conditions T₁-RNAase cleaves predominantly (up to 75-80%) in the anticodon of yeast tRNA⁰sp between residues G₃₄ and U₃₅; also to a fewer extent (20%) between residues G₇₃ and C₇₄ in the 3' extremity of the tRNA molecules generating the fragments A, B, and C (see Fig. 1) (22).

Experimental conditions were the following: yeast tRNA⁰sp 2 mg/ml (about 80 µM), T₁-RNAase at 4 units/mg RNA, 0.02M sodium cacodylate buffer pH 6.5 and 0.01M MgCl₂. After 70 minutes incubation at 4°C, the reaction was stopped by the addition of an equal volume of phenol saturated with the same buffer as above containing 2.5% (w/v) sodium dodecyl sulfate and 0.34% (w/v) saccharose.

After 30 minutes of vigorous shaking, the mixture was centrifuged and the RNA contained in the aqueous phase was collected by precipitation with ethanol, redissolved in 0.3M ammonium acetate and precipitated again with ethanol. Separation of the fragments was performed by electrophoresis on polyacrylamide slab gels in the following conditions: ethanol precipitation RNA samples were first dissolved into a minimum volume of 6M urea containing 0.025% xylene cyanol and 0.025% bromophenol blue (as electrophoretic markers); the solution was then loaded onto a slab gel (40x20x0.15) cm containing 13.5% (w/v) acrylamide crosslinked with 0.68% (w/v) bis-
acrylamide in 100mM Tris-Borate buffer, pH 8.3, 2.5 mM Na₂ EDTA and 8.3M urea. Electrophoresis was conducted at room temperature under 500-800 volts until the xylene cyanol had reached 5cm from the bottom of the gel. The RNA bands were detected by U.V. shadowing (23) and eluted from the gel slices according to the procedure of Maxam and Gilbert (24).

S₁-hydrolysis.

The nuclease-S₁ privileged sites of cleavage in yeast tRNA⁴asp are in the anticodon loop and in the 3'-terminus region. Experimental conditions were those of Wrede et al. (25) except that the pH for the reaction was 5.0. Yeast tRNA⁴asp at 2 mg/ml was first preincubated in the reaction mixture for 30 min. at 37°C prior the addition of the S₁-nuclease (500 units/mg of tRNA). After 5 minutes incubation at 37°C, the reaction was stopped by the addition of ATP (4 mM final) and of phenol saturated with 50 mM sodium acetate buffer at pH 5.0. The nucleic acid was recovered as described above. Among multiple cleavage products it was possible to recover from the polyacrylamide gel the following fragments: one major species (50-60% recovery) from (5'p)U₅ to U₃ (3'-OH), the fragment C in Fig. 1; and one minor species (2-3% recovery) from (5'p)U₁ to U₁₃ (3'-OH), not shown in Fig. 1.

Identification of the tRNA fragments.

The RNA fragments resulting from the above nuclease digestion were identified by their terminal nucleotides and by their nucleotide sequences using ³²P and labelling techniques (26). The fragments harbouring a 5'OH terminus were phosphorylated with ³²P orthophosphate. The reaction mixture was 0.3 μM in [γ³²P]-ATP (3000 Ci/m mole) and 3.8 μM in fragment to be labeled; 200 units/ml of T₄-polynucleotide kinase were used (24, 26-27). At the end of the incubation period (75 min. at 37°C) the reaction was stopped by inactivating the enzyme at 65°C for 3 minutes.

The fragments having a 3'OH terminus were labelled by ligating [5'³²P]-pCp following the procedure described in (28). Usually the reaction mixture was 1.6 μM in fragment; 0.1 to 0.4 μM in [5'³²P]-pCp, 120 μM in ATP; 100 units/ml of T₄-RNA ligase were used, no dimethyl-sulfoxide was added. Prior to further nuclease digestion, the labelled RNA fragments having ³²P (either at 5'OH or at 3'OH) were purified by electrophoresis in polyacrylamide gels (P.A.G.E.) under denaturing conditions. Depending on the size of the fragments, slab gels were 10% (70-80 nucleotides), 13.5% (30-45 nucleotides) or 20% (4-30 nucleotides) in polyacrylamide; the concentration of bis-acryla-
Fig. 1. Schematic procedure for cleavage and reconstruction of yeast chimeric tRNA\textsuperscript{Asp}. Letters (A) to (K) identify the different fragments or molecules used (see text).
mide being always 1/20th of that of acrylamide. The RNA bands were detected by autoradiography using X-ray films (Kodak X-Omat R film-1) and the RNA recovered as described above. The labelled RNA was exhaustively digested with nuclease-P1 (in order to obtain $^{32P}$ nucleosides) for 5' labelled fragments (29) and with nuclease-T2 (in order to obtain $^{32P}$ nucleosides) for 3' labelled fragments (18). The digestion products with 5' or 3' unlabelled nucleoside monophosphate as markers were separated by a two-dimensional cellulose thin layer chromatography (30) using isobutyric acid : NH4OH 0.5M (5:3 v/v) as solvent for the first dimension and concentrated HCl : isopropanol : H2O (15:70:15 v/v/v) for the second dimension. The nucleotide standards were localised by U.V. detection and the labelled nucleotides by autoradiography; their identification was done by comparison of their relative positions on the chromatogram with standard maps (31). Further identification of the RNA fragments was also performed by sequencing technique after partial digestion of the products and electrophoresis as described in Carbon et al. (32).

Construction of a chimeric tRNA$^{\text{Asp}}$ having an anticodon X$^{32P}$ UpC, where X = G, A, U or C.

The general strategy used in order to reconstitute a whole tRNA molecule, starting with the half molecules of yeast tRNA$^{\text{Asp}}$ as prepared above, was the following (see Fig. 1).

a/ All steps of ligation, phosphorylation and dephosphorylation were better carried out on the two half molecules after reannealing. The use of the fragment (c) terminated with a 3' phosphate group instead of fragment (b) terminated with the CpCpA$_{\text{OH}}$ is important because of the blocking role of the 3' phosphate for the addition of pNp catalysed by T$_4$-RNA ligase. Moreover the 5' phosphate group at position 1 of the protected fragment (d), a potential "donor group" becomes unreactive with the T$_4$-RNA ligase (28).

In addition degradation of the fragments by low levels of contaminating nucleases in some commercial preparation of T$_4$-RNA ligase were also considerably reduced. Usually equimolecular amounts of the two complementary half molecules were mixed together at 60°C for 3 min. at concentration of 3 to 11 μM in the buffer used for the next enzymatic reaction. The mixture was then allowed to cool at room temperature.

b/ Using such reannealed tRNA molecules (c), the fragment (d) terminated by U$_{35}$OH was elongated by one nucleotide (Np) using the same procedure as described above for the 3' end labelling. In this reaction,
the concentration of the reassocaited halves was 11 μM, the pNP being in a two fold excess with respect to the acceptor; the T₄-RNA ligase being at 150 units/ml. Very small amounts of [5' ³²P] pNP were added to the reaction mixture in order to follow the reaction. At the end of the incubation period (16 hours at 4°C), the reaction was terminated by heating at 60°C for 3 minutes. The RNA was then ethanol precipitated, dried under vacuum and dissolved in the buffer solution suitable for the next reaction step.

c/ Removal of the 3' terminal phosphate from either the fragment A, resulting from the T₄-RNA digestion of yeast tRNA<sub>Asp</sub> or of the above product elongated by Np(=P) was carried out by using the 3'-phosphatase activity of T₄-polynucleotide kinase (33-34). The products A + B annealed or G from previous reaction were redissolved at a concentration of 3 μM in 100 mM Imidazole-HCl buffer, pH 6.0 containing 10 mM MgCl₂, 10 mM β-mercaptoethanol, 0.02 mg/ml of bovine serum albumin and 200 units/ml of T₄-polynucleotide kinase for 2 hours at 37°C. The nucleic acids were recovered by ethanol precipitation and the dephosphorylated products H were isolated by electrophoresis on polyacrylamide gel (13.5% acrylamide in 8.3M urea) and eluted.

d/ Phosphorylation of the 5'OH terminus of the 3' half tRNA fragment B with radioactive [³²P] was carried out as described above, using T₄-polynucleotide kinase and 3.3 μM [γ³²P] ATP at 3000 Ci/mmole. After heat treatment of the mixture at 65°C for 3 minutes, the RNA fragment F was recovered by ethanol precipitation and reannealed with its complementary 5' half tRNA molecule (fragment H).

e/ The final step consists in covalent joining of the two half molecules by T₄-RNA ligase, under the same experimental conditions as used above for the 3' end labelling procedure giving molecule J or K. The concentration of the fragments was 1.6 μM and the amount of ligase was 13 units/ml. After 5 hours at 4°C, the reaction mixture was loaded on a 10% polyacrylamide gel and electrophorized in the presence of 8.3M urea. The reconstituted yeast tRNA<sub>Asp</sub> labelled in the anticodon with [³²P], was located on the gel by autoradiography; its position on the gel was compared with that of an authentic unlabelled yeast tRNA<sub>Asp</sub>, detected by U.V. shadowing. After elution of the tRNA, the solution was centrifuged to remove insoluble materials and the nucleic acid was precipitated twice with ethanol after redissolution in 0.3M sodium acetate.
Identifications on the final product.

In order to be sure that the tRNA was correctly sealed with the expected (Np) nucleotide at position 34 of the anticodon, aliquots of the labelled tRNA were exhaustively digested with nuclease-P1 or nuclease-T2. The digestion products were analysed by two dimensional chromatography on thin layer cellulose plates (30-31). The presence of a terminal adenosine was also controlled by 3' labelling with [5'32P] pCp, RNase-T2 digestion and subsequent monodimensional chromatography on thin layer cellulose plates.

Microinjection and maturation of tRNA in frog oocytes.

Adult Xenopus laevis females were obtained from the African Snake Farm at Fish Hoek, South Africa. Full grown oocytes (0.8 to 1.0 mm diameter) were dissected apart from the ovary and kept at 19°C in Barth solution (35). Microinjection was carried out essentially as described by Gurdon et al. (36-37) using 30 nl volume when microinjecting into the nucleus and 50 nl volume when microinjecting into the cytoplasm of the oocyte. tRNA to be microinjected was first dissolved in 50 mM Tris-HCl pH 7.6, 20 mM MgCl2, heated at 65°C for 3 min. and then renatured by slow cooling. The RNA was ethanol precipitated, dried under vacuum and finally redissolved in water at a concentration which gave about 30,000 to 500,000 cpm of [32P] per μl. The RNA solution was adjusted in order to microinject the same amount of nucleic acid into the nucleus or into the cytoplasm. All solutions to be microinjected were always centrifuged, using an Eppendorf microfuge, in order to remove any trace of insoluble materials. After injection, oocytes were incubated by groups of five in fresh Barth's medium at 19°C for a given period of time (as indicated in the figure 3). At the end of the incubation period, the oocytes were homogenized in 0.2 ml of 0.2M sodium acetate buffer (pH 4.5) containing 0.01M MgCl2; 0.001M Na2 EDTA and 1% (w/v) of sodium dodecylsulfate. The tRNAs were immediately extracted with 0.2 ml of phenol saturated in the same buffer. After centrifugation, the lower phase was reextracted with 0.2 ml of buffer. The tRNAs contained in the two pooled water phases were ethanol precipitated and subsequently purified by electrophoreosis on a 10% polyacrylamide gel under denaturing conditions. After elution of the nucleic acid from the corresponding bands, the amount of tRNA was estimated by counting the radioactivity by liquid scintillation. Identification of the labelled [32P] nucleotide located at the first position (wobble) of the anticodon was carried out after exhaustive RNase-T2 digestion. The procedure was the same as the one described above for the
analysis of the RNAase-\(T_2\) digestion except that the auto-radiography was
performed from 2 to 10 days using a Siemens intensifying screen. This led us
to detect amounts of radioactivity as low as 100 dpm. After exact location
on the T.L.C. plates, each of the labelled nucleotides was eluted with
water and its radioactivity determined by liquid scintillation. The radio-
activity found in each spot was expressed as the percentage of total radio-
activity found on the T.L.C. plate.

RESULTS AND DISCUSSION.

Enzymatic replacement, \textit{in vitro}, of the first anticodon base of yeast
aspartic-tRNA.

Using a combination of different enzymes: RNAase-\(T_1\), nuclease \(S_1\),
\(T_4\) - polynucleotide kinase and \(T_4\)-RNA ligase, under appropriate experimental
conditions, it was possible to prepare various fragments of yeast tRNA\(_{\text{Asp}}\).
These fragments were then used to reconstitute, \textit{in vitro}, chimeric tRNAs
harbouring any of the four normal nucleotides, i.e. G, A, U or C labelled
in its 3' position with \(^{32}\text{P}\) as the first nucleotide of anticodon, the
so-called "wobble" position. Fig. 1 illustrates the general strategy we
followed; all the experimental details being given in the previous section.

Provided all steps of ligation, phosphorylation and dephosphorylation
were carried out on the protected fragments, i.e. on two complementary half
molecules of tRNA previously reannealed together, the yield of each of the
individual steps (as determined by P.A.G.E. under denaturing conditions)
was quite satisfactory. Almost 90\% yield was obtained in each of the de-
phosphorylation steps and about to 60\% yield (depending on the excess of
ATP used) was encountered for the phosphorylation steps, both steps being
catalyzed by \(T_4\)-polynucleotide kinase. The alkaline phosphatase was never
used in our procedure because preservation of the terminal 5' phosphate
group of tRNA\(_{\text{Asp}}\) was a mandatory requirement.

The yield of the addition reaction of the mononucleoside 3', 5' diphosphate
pNp) on the \(D + C\) fragments reannealed was 80, 30, 20 and 10\% for pCp,
pAp, pUp and pGp respectively. However, instead of adding pGp onto frag-
ments \(D + C\) with \(T_4\)-RNA ligase, it appeared preferable to start with
purified fragment \(A\) harbouring \(G_{34}\). Reannealing steps, such as \(A + B\),
\(C + D\) and \(F + H\) were almost quantitative.

Sealing of the anticodon loop (steps \(E\) to \(J\) and \(F + H\)) gives \(K\) was
carried out with limiting amounts of \(T_4\)-RNA ligase. The yield of such a
reaction was in the range of 65 to 75%. Kaufmann and Littauer have obtained a 10% yield in the intermolecular joining reaction of two fragments of yeast tRNA^Phe; in this case however, the low yield was mainly due to the fact that only a minor fraction of the half tRNA molecules was reactive (12a) and not at all to inefficient cyclization reaction of the anticodon loop (even of six bases instead of seven in natural tRNA). (See also ref. 12b, 13 and 16).

One of the limiting factors in this methodology for producing large amounts of molecules J and K, happened to be the recovery step of the modified molecules from the polyacrylamide gels: in our hands, 50 to 60% only of the radioactive material was eluted from the RNA bands. Such drawbacks, however, should be overcome by the use of the high performance liquid chromatography technique.

In the process of producing molecule J, we currently obtained a yield of 50% when starting from equal amounts of purified fragments A + B. Lower yields were obtained in the preparation of molecule K; they appeared to be mostly depending on which pNP was being added to fragment D (see above).

An important improvement might be to make use of the enzyme tRNA nucleotidyl-transferase in order to add a CpCpAOH terminal sequence directly onto molecule G, after the dephosphorylation step, thus avoiding one P.A.G.E. step and reannealing of fragments H and P. Such a procedure was recently used by Bruce and Uhlenbeck at the final step of their modification procedure of anticodon loop in yeast tRNA^Phe (16). If the modified tRNAs are to be used for microinjection experiments (see below), such repair of the CCA end in vitro, however may not be essential because the tRNA nucleotidyl-transferase that is contained in frog oocyte is active on microinjected yeast tRNA lacking the CCA terminal (38), the only requirement being that the 3' terminal phosphate must be removed.

At the end of the whole procedure as described in fig. 1, extensive verifications were always performed by nearest neighbour analysis on the isolated products J and K. Fig. 2 shows that complete hydrolysis by T2-RNase liberated the expected \(^{3'-32P}\) labelled N\(_{34}\)p, while complete P\(_1\)-nuclease hydrolysis liberated the unchanged \(^{5'-32P}\) labelled pU\(_{35}\). In one case, beside the expected C\(_{34}\)p, a trace amount of \(^{3'-32P}\)-Up was also present (see fig. 2 and fig. 3). The reason was that for that particular experiment, fragment H was contaminated with fragment D, due to a too
short electrophoresis run between steps (G) and (H), giving rise to the formation of minor fractions of reconstituted tRNA\textsubscript{Asp} having six bases in the anticodon.

The whole procedure described above might be applied to any other tRNA harbouring selective cleavage sites in the anticodon loop. It was already successfully applied in reconstituting chimeric yeast tRNA\textsubscript{Arg} (normal anticodon ICG), yeast tRNA\textsubscript{Cys} (normal anticodon GCA), yeast tRNA\textsubscript{Leu} (normal anticodon UAG) and yeast tRNA\textsubscript{Met} (normal anticodon CAU). (Fournier, M., Vacher, J., Carbon, P., Beauchemin, N., unpublished results).

Post-transcriptional modification of chimeric yeast tRNA\textsubscript{Asp} after micro-injection into frog oocytes.

Taking advantage of the microinjection technique, we were able to accurately check the stability of the reconstituted \([^{32}\text{P}]\) labelled tRNA molecules (J) and (K) as well as the possible modifications (maturation) of the wobble nucleotide inside the cytoplasm or the nucleus of a given cell type, namely the frog oocyte. Compounds (E), (J) and (K) were microinjected into the cytoplasm of the oocyte. After increasing incubation periods at 19°C, they were recovered by phenol extraction and submitted to P.A.G.E. analysis. Results demonstrated that compound (E) quickly disappeared (after 4 to 6 hours, see fig. 3A-B), while no degradation of compounds (J) or (K) was evident, even up to at least

Fig. 2. Nearest neighbour nucleotides analysis of the \([^{32}\text{P}]\) in chimeric yeast tRNA\textsubscript{Asp} harbouring different anticodons. Autoradiography of monodimensional chromatography on T.L.C. plates of complete digested tRNA. Triplets denote the anticodons, the asterisk indicates position of the \([^{32}\text{P}]\) label. Conditions for hydrolysis and for chromatography are described in "Experimental procedures".
Fig. 3, Part A and B: Autoradiography of the polyacrylamide slab gel of compound © (in part A) and of compound Ø (in part B) incubated for different time (as indicated in the figure) inside the oocytes. Arrow © corresponds to position of intact molecule © prior to the microinjection; arrow Ø corresponds to fragment ©; arrow © is the unidentified degradation product of fragment ©; BB and XC are the positions of xylene cyanol of bromophenol blue marker dyes.

Fig. 3, part C: summarizes all the results obtained as in part A and B for compounds ©, © and ©, harbouring different combinations of anticodon. • for G[32p] UC, 13,000 cpm microinjected per oocyte; ■ for C[32p] UC, 11,000 cpm per oocyte; ¶ for U[32p] UC, 1,500 cpm per oocyte; ▲ for A[32p] UC, 1,900 cpm per oocyte; • anticodon unsealed i.e. with G34-OH and U35[32p] U35C, 10,000 cpm per oocyte.
45–72 hours of incubation. The same results were obtained when tRNA was microinjected into the nucleus of the oocytes (results non shown).

The degradation observed with compound E (anticodon unsealed) did not seem to be simply due to a phosphatase activity which would have eliminated the terminal $[\text{32P}]$ from the nucleic acid material. Indeed, free $[\text{32P}]$ orthophosphate was not found in band 3 in fig. 3A. As for RNAase-D in E. coli (39), it is tempting to postulate the existence of an exonuclease inside the oocyte which would act as a scavenger for some nonfunctional RNAs. Further investigations however are necessary in order to identify the hydrolysis product and to clarify this point.

High stability of microinjected tRNA, as well as of 5s-RNA and of messenger RNA but not of synthetic polynucleotides was already reported (40-41, reviewed in 42); our results show that it is also the case with chimeric tRNA carrying unusual anticodon such as those with A$_{34}$ (Figs. 3C).

More interesting was the observation that during the incubation of the tRNA (compound F) inside the oocyte, appreciable modifications of the G$_{34}$ and U$_{34}$ residues, in the first position of the anticodon, did occur. By contrast no modification was evident when the same position was occupied by either C$_{34}$ or A$_{34}$ in the anticodon of yeast tRNA$_{Asp}$.

Fig. 4 shows that after 24 hours of incubation, about 70% of the G$_{34}$ were replaced by Queuosine (Q$_{34}$) base, a derivative of G; moreover, a minor fraction (2%) of this Queuosine moved in the two dimensional cellulose thin layer plates as modified Q, probably mannosyl-Q (man-Q$_{34}$). Such glycosylated derivatives have been found in many other tRNA$_{Asp}$ of eukaryotic origin, but never in tRNA$_{Asp}$ of prokaryotes (43). Moreover, yeast tRNA never contain Q$_{34}$ base (43), most probably because the Q-insertase does not exist in such class of microorganisms. Yeast tRNA$_{Asp}$ retains however the intrinsic ability to react with Q-insertase of the frog oocyte (see also ref. 18) as well as with the same enzyme from E. coli (44). Thus clearly the informations recognized by the Q-insertase of the frog oocyte or of E. coli in order to process G$_{34}$ into Q$_{34}$ is present in yeast tRNA$_{Asp}$; more details on such molecular process will be published in a next paper.

No difference in the level of G$_{34}$ to Q$_{34}$ and man-Q$_{34}$ replacement was observed with yeast tRNA$_{Asp}$ (anticodon G$_{32}$P$^{[\text{32P}]_UC}$) microinjected into the cytoplasm or the nucleus (see fig. 4). Since no transfer of mature tRNA from the cytoplasm to the nucleus seemed to occur (45), our results indicate that the Q-insertase is probably cytoplasmic. However, we cannot excluded that a Q-insertase activity also exists inside the nucleus. Our results
Fig. 4. Autoradiography of the two dimensional T.L.C. plates of complete T2-digest of chimeric yeast tRNA$^{\text{Asp}}$ before and after microinjection into the frog oocyte. Triplets indicate the kind of anticodon, the asterisk indicates the position of the$^{[32p]}$ label: results correspond to tRNA harbouring G$^4$UpC, U$^4$UpC, C$^4$UpC and A$^4$UpC respectively. Time of incubation at 19°C and location of the microinjected product are given in the figure; control concerns the result with the chimeric yeast tRNA$^{\text{Asp}}$ before it was microinjected into the frog oocyte. On the upper left is the schematic drawing of the standard reference map used in order to tentatively identify the compounds.
clearly parallel the observation of Nishikura et al. (18–19) who micro-
injected into the nucleus of Xenopus laevis, the gene containing the
sequence of yeast tRNA\textsubscript{\textit{Yr}} (anticodon G\textsubscript{\textit{Yr}}). In this case, one of the last
steps of the transcription and maturation processes was the G\textsubscript{34} to Q\textsubscript{34}
modification and galactosyl-Q\textsubscript{34} transformation to an extent of about 20–30% (Q\textsubscript{34} + gal-Q\textsubscript{34}).

With microinjected chimeric yeast tRNA\textsubscript{Asp} harbouring the anticodon U[\textsuperscript{32p}]UC, (corresponding to codons CA\textsubscript{G} for glutamic acid), two new spots of different relative intensity (respectively 70% and 3% of the total radioactivity on the T.L.C. plates) were detected (fig. 4). According to Waldron et al. (46), these spots correspond to mcm\textsubscript{5}\textsuperscript{2}U and mcm\textsubscript{5}U. These modified bases were also found in several tRNAs, especially in yeast tRNA\textsubscript{Glu} (anticodon mcm\textsubscript{5}\textsuperscript{2}UUC) and yeast tRNA\textsubscript{Lys} (anticodon mcm\textsubscript{5}\textsuperscript{2}UUC) (43). To ascertain the nature of the modified U\textsubscript{34} in chimeric yeast tRNA\textsubscript{Asp}, cochromatography experiments involving T\textsubscript{c}-RNase complete digest of authentic yeast tRNA\textsubscript{Glu} and of Xenopus laevis tRNA\textsubscript{Glu} are however necessary. This would lead to demonstrate that the information recognized by the modifying enzyme for processing U\textsubscript{34} into mcm\textsubscript{5}\textsuperscript{2}U\textsubscript{34} is present in both chimeric yeast tRNA\textsubscript{Asp} and Xenopus laevis tRNA\textsubscript{Glu}.

With chimeric yeast tRNA\textsubscript{Asp} harbouring anticodon C[\textsuperscript{32p}]UC and A[\textsuperscript{32p}]UC, no modification of the nucleoside in the first position of the anticodon occurred when they were microinjected into the cytoplasm or the nucleus. Such a result with C\textsubscript{34} in chimeric tRNA\textsubscript{Asp} is not surprising because we already know that most tRNAs of eukaryotic origin have no modified C\textsubscript{34} except for tRNAs specific for leucine (anticodons m\textsuperscript{5}CAA and CmAA) and for tryptophan (anticodon CmCA) (43).

The fate of A\textsubscript{34} is a puzzling problem. It is generally believed that an enzyme catalyses the transformation of A\textsubscript{34} into Inosine (I\textsubscript{34}) by an oxidative deamination process (47–50). With chimeric yeast tRNA\textsubscript{Arg}\textsubscript{\textit{I}} (anticodon A[\textsuperscript{32p}]GC instead of the normal ICG) such a transformation of A\textsubscript{34} into I\textsubscript{34} indeed occurred in the cytoplasm and the nucleus of the Xenopus laevis oocyte (Pournier, M., unpublished results). Insofar as the oocyte modifying enzyme can also interact with the chimeric yeast tRNA\textsubscript{Asp} (anticodon A[\textsuperscript{32p}]UC, our result would suggest that the presence of an A in position 34 is not a sufficient condition to cause its transformation into Inosine.
CONCLUSIONS.

The main purpose of the present work was to change the nucleotide at position 34 (the so called 'wobble' position) as well as to introduce there a $^{32}P$ label in yeast tRNA$^{Asp}$ and to look for the possible modifications of such chimeric tRNA inside the cytoplasm or the nucleus of the oocyte of Xenopus laevis.

Such a methodology provides valuable information on the nature and the extent of a given post-transcriptional modification of the tRNA molecule. It also gives information about the cellular location of the modification machinery. It should also bring about new ways of studying the specificity of interaction between a modifying enzyme and a tRNA or the new coding properties of chimeric tRNAs under in vivo conditions.

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FOOTNOTES.

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** Abbreviations: A, adenosine; C, cytosine, G, guanosine; U, uridine; I, inosine; N, any of the four nucleotide A, C, G or U; Q, 7-(4,5 cisdi-hydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine; mcm$^5$U, 5-methoxycarbonylmethyluridine; mcm$^5$s$^2$U, 2-thio-5-methoxycarbonylmethyluridine; m$^2$C, 5-methylcytidine; Cm,2'-O-methylcytidine; T.L.C., thin layer chromatography; P.A.G.E., polyacrylamide gel electrophoresis.

*** The numbering system of nucleotides in tRNA$^{Asp}$ is the one adopted at the 1978 Cold Spring Harbor Meeting on tRNA, New York, 1978 (see also reference 43.)

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